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BeWo cells stimulate SMC apoptosis and elastin breakdown in a model of spiral artery transformation

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Abstract

BACKGROUND: During pregnancy, extravillous trophoblast invades the uterine wall and enters the spiral arteries. Remodelling ensues, with loss of vascular smooth muscle cells (SMC) to create high flow, low resistance vessels. Pregnancies complicated by pre-eclampsia are characterised by incomplete arterial remodelling. Endovascular trophoblast is not easily accessible for studies to establish the pathogenesis of pre-eclampsia so we have developed a model appropriate to carry out mechanistic studies of vessel wall transformation. METHODS AND RESULTS: Segments of human spiral artery were perfused with the choriocarcinoma cell line BeWo; cells invaded the vessel wall and induced apoptosis of vascular SMC. Perfusion of vessels with BeWo-conditioned medium also induced SMC apoptosis, indicating the presence of a soluble apoptotic factor. BeWo express Fas ligand (FasL) and Tumor Necrosis Factor-related Apoptosis-Inducing Ligand (TRAIL). Treatment of BeWo-conditioned medium with antibodies against FasL inhibited vascular SMC apoptosis *in vitro*. Antibodies that blocked TRAIL receptor function had no effect. Extracellular matrix degradation is also a prerequisite for vascular remodelling; BeWo express matrix metalloproteinase-12 (MMP-12) and BeWo-conditioned medium increased MMP-12 expression in spiral artery SMC. CONCLUSIONS: BeWo induce arterial remodelling via FasL- and MMP-12-dependent mechanisms. BeWo-derived factors upregulate protease expression in spiral artery SMC to facilitate matrix breakdown.

Introduction

Maintenance of a successful pregnancy is dependent on the ability of the trophoblast to invade the uterus and remodel the spiral arteries. The process of remodelling, known as physiological change, converts these vessels into high flow, low resistance conduits that lack maternal vasomotor control (Benirschke *et al.,* 2000; Kam *et al.,* 1999; Pijnenborg *et al.,* 1983; Pijnenborg *et al.,* 2006). This transformation is necessary to meet the increasing demands of the developing fetus for blood and oxygen.

Early in pregnancy, extravillous trophoblast detaches from cell columns (Aplin 1991), and invades the uterine wall via one of two routes. Interstitial trophoblast invades the uterine stroma as far as the first third of the myometrium, while endovascular trophoblast enters the spiral arteries and migrates to a similar depth, in a retrograde manner. The combined actions of these two cell populations leads to spiral artery transformation (Kam *et al.,* 1999; Pijnenborg *et al.,* 1983). Vessel remodelling involves a partial loss of endothelium, and replacement of the internal elastic lamina and musculo-elastic media with an amorphous fibrinoid material in which trophoblast is embedded (Brosens *et al.,* 1967; Pijnenborg *et al.,* 1983). Diseases of pregnancy such as pre-eclampsia, fetal growth restriction and second trimester miscarriage are all associated with impaired arterial remodelling. Shallow trophoblast invasion, decreased numbers of invasive trophoblasts (Khong *et al.,* 1986; Naicker *et al.,* 2003) and the absence of endovascular trophoblast from the myometrial segments of spiral arteries have all been observed in pre-eclampsia (Kadyrov *et al.,* 2003). Spiral artery remodelling is also reduced or absent in hypertensive diseases of pregnancy (Pijnenborg *et al.,* 1991), in the maternal uterus of small-for-gestational-age infants (Khong *et al.,* 1986) and in late sporadic miscarriage (Ball *et al.,* 2006).

We and others have previously shown that first trimester cytotrophoblasts induce apoptosis of vascular cells during arterial remodelling (Ashton *et al.,* 2005; Cartwright *et al.,* 2002; Crocker *et al.,* 2005; Red-Horse *et al.,* 2006). When introduced into the lumen of isolated human spiral artery segments, these cells colonise the vessel wall and induce endothelial and smooth muscle cell (SMC) death. To do this, trophoblasts utilise members of the Tumor Necrosis Factor (TNF) family of apoptosis-inducing ligands. First trimester cytotrophoblasts release soluble Fas ligand (Abrahams *et al.,* 2004; Harris *et al.,* 2006), which effects apoptosis following ligation of the Fas receptor on arterial endothelial cells and SMC (Ashton *et al.,* 2005). They also express Tumor Necrosis Factor-related Apoptosis Inducing Ligand (TRAIL), a membrane bound ligand which binds to TRAIL Receptor-1 (TRAIL-R1) and TRAIL Receptor-2 (TRAIL-R2) present on arterial SMC (Keogh *et al.,* 2007). Along with trophoblast-derived proteases and cytokines present in the uterine environment, these ligands regulate the complex process of arterial transformation.

The BeWo cell line, derived from human gestational choriocarcinoma, has been widely used as a model of trophoblast (Church *et al.,* 1998; Egawa *et al.,* 2002; Ishimatsu *et al.,* 2005; Li *et al.,* 2003). As the availability of systems to study physiological change is limited, we have examined whether BeWo can remodel spiral artery segments in a manner similar to endovascular trophoblasts. We have also studied the mechanisms they employ to induce apoptosis of vascular SMC.

Materials and methods

Reagents

Reagents were purchased from the following sources: mouse anti-human Fas (CD95) monoclonal antibody, mouse anti-human MMP-12 monoclonal antibody (catalytic domain), mouse anti-human MMP-12 monoclonal antibody (hemopexin domain), recombinant human MMP-12 western blotting standard, rabbit anti-human active caspase-3 polyclonal antibody, R&D Systems (Minneapolis, MN, USA); mouse anti-human FasL monoclonal antibody (clone NOK-1), mouse anti-human FasL monoclonal antibody (clone NOK-2), BD Pharmingen (San Diego, CA, USA); mouse IgG2a κ (clone UPC-10), rabbit anti-human cleaved poly-ADP-ribose polymerase (PARP, p85 fragment) polyclonal antibody, Promega Corporation (Madison, WI, USA); mouse anti-human cytokeratin-7 monoclonal antibody (clone OV-TL 12/30), rabbit anti-mouse biotinylated antibody, swine anti-rabbit biotinylated antibody, streptavidin-FITC, FITC-conjugated rabbit anti-mouse IgG, HRP-conjugated goat anti-mouse IgG, FITC-conjugated swine anti-rabbit IgG, DakoCytomation A/S (Glostrup, Denmark); mouse anti-human CD34 monoclonal antibody, Serotec (Oxford, UK); mouse anti-human TRAIL monoclonal antibody, mouse anti-human TRAIL receptor-1 monoclonal antibody, mouse anti-human TRAIL receptor-2 monoclonal antibody, Alexis Biochemicals (distributed by Axxora, Nottingham, UK); in situ cell death detection kit (TUNEL), Roche (Lewes, UK); annexin V-FITC apoptosis detection kit, BD Pharmingen (Oxford, UK); Vectashield mounting medium, Vector Laboratories Inc. (Burlingame, CA, USA); OCT embedding medium, Raymond A Lamb (London, UK); CellTracker CM-Dil, Molecular Probes (distributed by Invitrogen Ltd, Paisley, UK); tissue culture medium, Cambrex (Wokingham, UK); fetal bovine serum, Gibco (distributed by Invitrogen Ltd, Paisley, UK); Matrigel, BD Discovery Labware (Bedford, MA, USA); collagen I,

collagen IV, elastin, fibronectin, laminin, Sigma-Aldrich Inc. (Saint Louis, MO, USA); Rainbow™ molecular weight markers, Hybond™-P PVDF membrane, ECL Plus western blotting detection reagents, Amersham Biosciences UK Ltd. (Chalfont St.Giles, UK); Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich and were of AnalR grade.

Tissue

Informed consent was obtained for all myometrial and placental tissue used in this study, and local ethical committee approval was in place. Normal first trimester placenta (8-12 weeks gestation) was obtained at elective termination of pregnancy (surgical or medical). Umbilical cords were obtained from normal term placentae within 30 minutes of caesarean section or vaginal delivery. Term decidual/myometrial biopsies taken from non-placental bed tissue were obtained from women with normal pregnancies at elective caesarean section.

Vessel explant model

Dissection and perfusion of spiral arteries were performed as previously described (Cartwright *et al.*, 2002; Cartwright *et al.*, 2006). Briefly, unmodified spiral arteries were dissected from term decidual/myometrial biopsies under sterile conditions and mounted on glass cannulae in a pressure myography perfusion chamber (Living Systems Instrumentation, Burlington, VT, USA). Arteries were denuded of endothelium by passing a column of air through the vessel and then perfused with the appropriate medium or cells, as indicated. The ends of each vessel were tied and the arteries incubated for up to 72 h in 1:1 DMEM:Ham's F12 culture medium supplemented with 10% FBS, glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml).

Umbilical artery segments were dissected under sterile conditions from umbilical cords and cannulated with a needle and syringe. Sterile PBS was forced through the segments to

remove the endothelium. Arteries were then perfused with the appropriate medium or cells, as indicated. The ends of each vessel were tied and the arteries incubated for up to 96 h in 1:1 DMEM:Ham F12 culture medium supplemented with 10% FBS, glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml).

Cell culture and labelling

BeWo cells were cultured in 1:1 DMEM:Ham F12 culture medium supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml). The human aortic SMC (HASMC) cell line was cultured as previously described (Harris *et al.*, 2006; Keogh *et al.*, 2007) and maintained in Kaighn's modification of Ham F12 medium supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml). All cells were incubated with 95% air and 5% carbon dioxide at 37°C in a humidified incubator.

Co-culture assay

Proliferating HASMC were labelled with CellTracker (1µg/ml) in serum-free medium for 5 minutes at 37°C, followed by 15 minutes at 4°C. HASMC were washed twice in PBS and transferred back to serum-containing medium, before BeWo cells were added to the SMC, at a ratio of approximately 1:1. Cells were maintained in a 1:1 ratio of SMC medium:BeWo medium. After 48 h, adherent and non-adherent cells were collected. Cells were centrifuged, the supernatant discarded and the pellets were analysed for phosphatidylserine externalisation (to detect SMC apoptosis; described below). The number of apoptotic SMC (positive for both CellTracker (red) and annexin V (green)) was quantified by flow cytometry.

For experiments requiring the addition of blocking antibodies, SMC were labelled with CellTracker as described above and transferred back into serum-containing medium. BeWo were added and allowed 2 h to adhere. After this time, cultures were washed twice in PBS and maintained in serum-free medium (1:1 ratio of SMC medium:BeWo medium). TRAIL receptor blocking antibodies (final concentration 5µg/ml) were also added at this time. After 48 h, the number of SMC externalising phosphatidylserine in each co-culture was quantified by flow cytometry, as described below.

Conditioned medium and blocking antibody treatments

BeWo conditioned culture medium was produced in the presence or absence of serum by culturing BeWo cells in a T_{75} flask with 15 ml of medium for 48 h. Conditioned medium was diluted 1:1 (v/v) with unconditioned medium before use. For flow cytometry experiments using the FasL blocking antibody NOK-2, serum-free unconditioned medium (control) or BeWo-conditioned medium was incubated with NOK-2 (10 µg/ml) or an isotype-matched control antibody IgG2a κ (10 µg/ml) for 1 h at room temperature. HASMC were then incubated with the depleted medium for 2 h, also in the absence of serum.

Immunohistochemistry

After culture, vessels were fixed with 2% (v/v) paraformaldehyde in PBS for 30 min and then incubated with 0.5 M sucrose in PBS for at least 1 h. The tissue was placed in OCT embedding medium, frozen and stored at -80°C. Transverse sections (10 µm) of frozen vessels were prepared using a cryostat, transferred to poly-L-lysine-coated slides and stored at -80°C. For immunostaining of vessel sections, slides were warmed to room temperature and fixed with 4%

(v/v) paraformaldehyde for 20 min. For immunostaining of BeWo, cells were cultured on glass coverslips and fixed with 4% (v/v) paraformaldehyde for 20 min. Following a washing step in PBS, both cover slips and tissue sections were permeabilised with 0.1% (v/v) Triton-X in PBS for 5 min, washed in PBS and allowed to air dry before addition of the primary antibody. Primary and secondary antibodies were diluted in PBS and applied to the tissue or cover slip for 1 h, during which the slides/cover slips were placed in a humidified chamber at room temperature. Samples were protected from light once the secondary antibody was applied. Following each antibody incubation, the slides or cover slips were washed with 3 times in PBS. Antibody dilutions used were as follows: active caspase-3 (0.25 µg/ml), CD34 (1:10), cleaved PARP (1:100), cytokeratin-7 (1:40), Fas ligand (NOK-1; 1:50), MMP-12 (1:20), TRAIL (1:20), FITC-conjugated rabbit anti-mouse and swine anti-rabbit secondary antibodies (1:40), biotinylated mouse-anti rabbit (1:200) and biotinylated rabbit-anti swine secondary antibodies (1:500), and streptavidin-FITC (1:100). Sections and cover slips were mounted using Vectashield mounting medium containing propidium iodide and stored at 4°C in the dark until viewed. Slides were analysed at room temperature using either an Olympus IX70 inverted fluorescence microscope or a Biorad Radiance 2100 confocal microscope with a 10x or a 40x oil immersion objective lens and LaserSharp 2000 image analysis software.

A modified protocol was used to stain BeWo cells for Fas ligand or TRAIL. After incubation with primary antibody and subsequent PBS washes, cells were incubated with a biotinylated secondary antibody for 1 h. Following further washes, streptavidin-FITC was applied for 1 h, cells were washed 3 times in PBS and mounted as previously described.

TUNEL staining

Vessel sections were fixed using 4% (v/v) paraformaldehyde in PBS for 20 min at room temperature, washed in PBS for 20 min and allowed to air dry. Slides were then incubated with permeabilisation solution (0.1% (v/v) Triton in 0.1% (w/v) sodium citrate in H_2O) for 8 min and washed in PBS. Slides were allowed to air dry before incubation with 16 µl of TUNEL reagent per tissue section. The working TUNEL reagent solution was prepared according to the manufacturer's instructions, although the TUNEL enzyme provided was diluted 1:5 with PBS to reduce background fluorescence. Slides were incubated in a humidified chamber for 1 h in the dark, then washed in PBS. Slides were mounted using Vectashield mounting medium containing propidium iodide and stored at 4°C in the dark. Quantification of the number of TUNEL-positive cells per vessel section was performed blind, using an Olympus IX70 inverted fluorescence microscope. TUNEL-positive cells present in the two layers of cells closest to the lumen were excluded to omit residual endothelial cells or adherent trophoblast from the counts. A minimum of 6 sections were stained and counted per vessel.

Annexin V assay

Phosphatidylserine externalisation was quantified by flow cytometry, using a commercially available annexin V-FITC apoptosis detection kit following the manufacturer's guidelines. Following treatment, HASMC were washed twice in PBS, trypsinised and collected. The culture medium was also retained and pooled with the adherent cells. Cells were centrifuged, the supernatant discarded and the cell pellets resuspended in kit binding buffer. The cells were centrifuged again, the supernatant discarded and the pellet re-suspended in kit buffer (100 µl/pellet) containing annexin V solution (5 µl/pellet) and propidium iodide (2.5 µg/ml). Samples

were incubated in the dark for 10 min and the percent of annexin V-positive cells was analysed using a Coulter Epics Elite flow cytometer.

TRAIL expression

Proliferating BeWo cell monolayers were washed twice in PBS, trypsinised and collected in serum-containing medium. Cells were centrifuged, the supernatant discarded and the pellets washed three times in PBS containing BSA (0.5%) and NaN_3 (0.1%). Cells were incubated on ice with a mouse anti-human TRAIL antibody (25µg/ml) or a control IgG2 (25µg/ml) for 1h. Cells were then washed 3 times in PBS/BSA/NaN_3 and incubated with a rabbit-anti mouse FITC-conjugated secondary antibody (1:40 dilution) for 1h in the dark. Cells were again washed 3 times in PBS/BSA/NaN_3 and the percent of fluorescent cells was quantified using flow cytometry.

Western blotting

HASMC were washed with PBS, incubated on ice in lysis buffer (1x PBS, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM Na_3VO_4, 1 mM PMSF, 10 ▬g/mL aprotinin), scraped from dishes, centrifuged and the lysates retained for analysis. Equal amounts of protein were separated by SDS-PAGE on 10% gels and transferred to PVDF membranes. Membranes were blocked (Tris-buffered saline (TBS), 0.1% (v/v) Tween, 5% (w/v) milk powder), and then probed with mouse anti-human MMP-12 antibody (1:200) prepared in TBS-Tween (3% (w/v) BSA), followed by an HRP-conjugated secondary antibody (1:1000). Following washing, proteins were detected by enhanced chemiluminescence.

Statistics

Data were compared using either a repeated measures ANOVA or paired *t* test (parametric) or a Kruskal-Wallis test (non-parametric). Appropriate post-hoc tests were applied and all statistical analyses performed using GraphPad Prism software, version 4 (GraphPad Software, San Diego, CA, USA). Significance was taken as $p<0.05$. Data are presented as the mean ± SEM from at least 3 independent experiments.

Results

Using an *ex vivo* model of endovascular trophoblast invasion, we have studied the ability of BeWo to invade segments of human spiral artery and induce apoptosis of medial SMC. To facilitate rapid access to the SMC layers, vessel segments were denuded of endothelium prior to perfusion. Removal of the arterial endothelium was confirmed by CD34 staining (data not shown). Forty eight hours after introduction into the lumen of spiral artery segments, cytokeratin-7 positive cells could clearly be observed in the vessel wall (Figure 1A), although the extent of invasion was quite variable, with some regions more highly colonised than others. Areas of SMC apoptosis were less heterogeneous; subpopulations of TUNEL-positive cells were observed throughout the arterial segments following perfusion with either BeWo cells or their conditioned medium (Figure 1C, D). In contrast, control vessels exhibited minimal SMC apoptosis (Figure 1B). Immunohistochemistry using antibodies against active caspase-3 and cleaved poly-ADP-ribose polymerase (PARP) confirmed that SMC death was occurring by apoptosis (data not shown).

Although the number of TUNEL-positive SMC observed within arteries was increased after the introduction of cells, this did not reach statistical significance (Figure 2A). However, BeWo-conditioned medium induced SMC apoptosis significantly above control levels at 24h, 48h and 72h ($P<0.05$). Denuded segments of umbilical artery, which contain many more layers of SMC, were also perfused with BeWo cells (Figure 2B). This resulted in a significant increase in the number of TUNEL-positive SMC after 48h ($P<0.05$). Conditioned medium also increased apoptosis of umbilical artery SMC after 24h ($P<0.05$), 48h ($P<0.05$) and 96h ($P<0.01$) in culture. These data suggest that pro-apoptotic mediators are secreted by BeWo cells. Fas ligand and TRAIL are utilised by first trimester cytotrophoblasts to induce SMC apoptosis during spiral

artery remodelling (Harris *et al.*, 2006; Keogh *et al.*, 2007). A majority of BeWo cells expressed surface Fas ligand (Figure 3A), 68.4 ± 3.5 % (mean ± SEM, n=3; flow cytometry). In contrast, surface TRAIL expression was only detected on 15.4 ± 5.2% (mean ± SEM, n=4; flow cytometry). Confocal microscopy confirmed these findings and revealed that TRAIL is present in vesicles within the cytoplasm of some, but not all, cells (Figure 3B).

To further study the soluble pro-apoptotic factors released by BeWo cells, a human aortic SMC cell line was cultured in BeWo-conditioned medium (Figure 4A), and apoptosis was quantified using phosphatidylserine externalisation measured by flow cytometry. SMC apoptosis was significantly increased after treatment with conditioned medium, with approximately 30% of SMC externalising phosphatidylserine after 2 h, 4 h and 24 h ($P<0.001$) in culture. To test for soluble Fas ligand, serum-free conditioned medium was pre-treated with Fas ligand-blocking antibody or control IgG. Conditioned medium containing control IgG increased the number of apoptotic SMC significantly above levels observed in control cultures ($P<0.01$; Figure 4B). When the conditioned medium was pre-treated with a Fas ligand-blocking antibody, levels of SMC apoptosis were significantly decreased ($P<0.01$).

To investigate whether TRAIL, which exists predominantly as a membrane-bound ligand, induces SMC apoptosis, a dual culture system was employed in which fluorescently labelled SMC make direct contact with BeWo. After 48h, approximately 25% of SMC were apoptotic, in contrast to approximately 5% in monoculture (Figure 5A). This experiment was repeated in the presence of antibodies that blocked TRAIL-R1 and TRAIL-R2. As previously observed, the presence of BeWo cells significantly increased the percent of SMC externalising

phosphatidylserine ($P<0.01$; Figure 5B), although due to the absence of serum, the baseline level of apoptosis was increased. The addition of TRAIL receptor blocking antibodies had no effect on the level of SMC apoptosis observed in the co-cultures.

The specialised ECM environment of the vessel wall may affect target cell susceptibility to apoptosis. SMC were cultured in the absence of serum on culture plates which were either uncoated or coated with collagen I, collagen IV, elastin, Matrigel, fibronectin or laminin. Cells were stimulated with a Fas-activating antibody and after 48h, phosphatidylserine externalisation was quantified. At this time, approximately 30% of SMC grown on uncoated plates were apoptotic; this figure rose to 55% when SMC were challenged with a Fas-activating antibody (Figure 6). Surprisingly, none of the matrix components tested offered any protection against Fas-induced apoptosis.

To successfully remodel the spiral arteries, trophoblasts must degrade extracellular matrix components, including elastin fibres within the medial SMC layers. BeWo express the elastolytic enzyme matrix metalloproteinase-2 (MMP-2) (Di Simone *et al.*, 2006; Mandl *et al.*, 2006) but express little or no MMP-9 (Mandl *et al.*, 2006; Morgan *et al.*, 1998). In this study we assessed expression of the elastase MMP-12 (macrophage metalloelastase) by BeWo using immunohistochemistry and western blotting. BeWo predominantly expressed the active 45kDa form of MMP-12 (Figure 7A), which exhibited a punctuate distribution within the cytoplasm (Figure 7B).

During the invasion process, trophoblast may promote protease expression in spiral artery SMC. To investigate this, we cultured human aortic SMC *in vitro* with BeWo-conditioned medium for

24h and measured the expression of MMP-12 by immunoblotting. Levels of active MMP-12 were not significantly increased following treatment with BeWo-conditioned medium (Figure 8A), nor did induction of SMC apoptosis using a Fas-activating antibody or etoposide alter MMP-12 expression. However, expression of MMP-12 was increased in the medial SMC layer of spiral arteries perfused with BeWo conditioned medium (Figure 8C). Vessels perfused with control medium exhibited negligible staining (Figure 8B).

Discussion

At present, the complex processes which regulate vessel transformation are poorly understood, primarily due to the lack of suitable cellular and tissue models in which to study this phenomenon. It is possible to observe arterial remodelling in human decidual/myometrial biopsies, but histology only supplies a snapshot of the process. Most rodent systems are limited by the lower level of trophoblast invasion, the later stage of pregnancy at which it occurs (Adamson *et al.,* 2002) and greater role in vascular transformation ascribable to uterine NK cells (Ashkar *et al.*, 2001). Trophoblast invasion and arterial remodelling has also been studied by transplanting human first trimester placental villi into mammary fat pads or beneath the kidney capsules of Scid mice (Red-Horse *et al.,* 2006). Data from these studies confirm a role for trophoblast-induced endothelial cell and SMC apoptosis in the process of vascular transformation. To tackle the problem from a different angle, our laboratory has developed an *ex vivo* model of endovascular trophoblast invasion and spiral artery remodelling where trophoblast is introduced into human spiral artery segments that can be maintained at high viability for several days (Cartwright *et al.,* 2002; Cartwright *et al.,* 2006).

In this study, we have examined the ability of BeWo cells to invade and remodel segments of spiral artery. BeWo cells exhibit many of the characteristics of invasive trophoblast: morphologically they are mononucleate and they secrete small amounts of placental alkaline phosphatase and human chorionic gonadotrophin (Friedman *et al.,* 1979). Akin to endovascular trophoblast, BeWo adhered to the vessel wall and penetrated the smooth muscle layers beneath. However, regions of invasion were highly variable; several intramural cells could be observed in some vessel sections, yet adjacent sections contained none. This may explain why conditioned culture medium but not the cells themselves induced SMC apoptosis in perfused vessel segments,

a result which indicates the release of soluble pro-apoptotic factors. It is possible that secretion occurs only after cells have attached to the vessel wall, generating a high local concentration of factor(s). Vessels perfused with conditioned medium from adherent cultures probably achieve a more uniform exposure to the apoptotic factors, resulting in an increased overall level of apoptosis.

In common with first trimester cytotrophoblasts (Harris *et al.,* 2006), BeWo cells employ Fas ligand to induce SMC apoptosis; however, unlike primary cytotrophoblast (Keogh *et al.,* 2007), they do not appear to utilise TRAIL, perhaps because of its relatively low level of expression on the cell surface. The heterogeneity of BeWo cell invasion may reflect this fact, or alternatively, the absence of the endothelium from the arterial segments may have hindered the initial stages of adhesion. We find this possibility unlikely however, as the extent of BeWo invasion in several vessels that contained remnants of endothelium was no different to the level of invasion observed in denuded vessels.

We also investigated whether components of the extracellular matrix have the ability to enhance cell survival, such that trophoblast-mediated breakdown of matrix components may promote SMC loss during vessel remodelling. This did not prove to be the case. Despite this, catabolism of extracellular matrix is still required to effect vessel transformation. Elastin fibres within the arterial media, and the internal elastic lamina present in myometrial vessels must be degraded to facilitate a permanent increase in vessel diameter and to abolish vasomotor control. BeWo cells express high levels of the elastase MMP-2 (Di Simone *et al.,* 2006; Mandl *et al.,* 2006), but negligible MMP-9 (Mandl *et al.,* 2006; Morgan *et al.,* 1998). Here we show that they also

express the 45kDa active form of MMP-12, which has documented elastolytic activity (Shapiro *et al.*, 1993).

In addition to trophoblast, vascular SMC may also play a role in elastin breakdown. Elastases including cathepsin S and K are upregulated by vascular SMC in mouse models of atherosclerosis (Cheng *et al.*, 2004), thus elastase expression may be increased in spiral artery SMC during the remodelling process. Here we show that SMC cultured in vitro express active MMP-12 and that the level of protein expression does not change following treatment with BeWo conditioned culture medium. In contrast, spiral artery SMC in situ do not express MMP-12 until they are challenged with BeWo-conditioned medium. Cultured SMC may constitutively express MMP-12 because their phenotype is more similar to that of spiral artery SMC exposed to trophoblast-derived factors. Nevertheless, these data serves as a reminder that experiments performed using cell lines must be repeated using primary cells/tissue. Taking these findings into account, we propose that trophoblasts release soluble factors which increase protease expression and/or activity in vascular SMC. These proteases can then act locally to degrade matrix components within the vessel wall, allowing easier access to invading trophoblasts. Thus, trophoblast may facilitate their own invasion by inducing cooperative breakdown of elastin fibres by medial SMC.

Studies of the placental bed have shown that spiral artery remodelling is a gradual, multi-step process that occurs throughout the first and second trimesters of pregnancy (Benirschke *et al.*, 2000). Consequently, this phenomenon must be tightly regulated to prevent sudden loss of vessel integrity. It is likely that a number of mechanisms act concurrently to facilitate vessel transformation, and that the process involves factors derived from the invading trophoblast, the

decidualised endometrium, resident leukocytes and the maternal endocrine system. There is evidence to suggest that changes in vascular structure, such as arterial dilation and disorganisation of the smooth muscle layers, occur prior to the arrival of extravillous trophoblast (Craven *et al.,* 1998). Changes such as replacement of the endothelium and SMC with trophoblast embedded in fibrinoid, only occur in the presence of trophoblast (Pijnenborg *et al.,* 1983). Dedifferentiation of vascular SMC and breakdown of mural extracellular matrix by trophoblast- and SMC-derived proteases are other features of physiological change that require further investigation.

Our current findings demonstrate that following introduction of BeWo cells into arteries, SMC apoptosis occurs slowly and in a highly controlled manner. This is essential if vessel integrity is to be maintained and is representative of the changes observed *in vivo*. Although we demonstrate here that BeWo-derived Fas ligand mediates SMC apoptosis, our results do not exclude the possibility that BeWo may also stimulate resident vascular SMC to produce apoptotic factors. Invading cells may promote the expression and/or release of Fas ligand by neighbouring SMC, so that cell death is regulated in a paracrine manner. However, the present study has shown that BeWo cells can induce apoptosis of spiral artery SMC via release of soluble Fas ligand, via a mechanism similar to that of primary trophoblast (Harris *et al.,* 2006). Genetic manipulation of BeWo prior to vessel perfusion may be useful in delineating key molecules involved in trophoblast invasion. We therefore propose this as an experimental model system to allow further investigation of vessel transformation *in vitro*.

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Figure legends

Figure 1: BeWo cells induce apoptosis of arterial smooth muscle cells *in situ*

A) Spiral artery (minus endothelium) perfused with BeWo for 48 h and stained with an antibody against cytokeratin-7 (green). B-D) Spiral artery (minus endothelium) perfused with B) unconditioned trophoblast medium (control), 48 h; C) BeWo cells, 48 h; D) BeWo-conditioned medium (50% (v/v)), 48 h. TUNEL-positive cells are labelled with FITC (green) and nuclei are counterstained with propidium iodide (red). * denotes the vessel lumen. Scale bar = 100µm. Pictures are representative of n≥3 independent experiments, with each experiment performed on vessels from a different biopsy.

Figure 2: BeWo-induced SMC apoptosis in an *ex vivo* vessel model

Spiral arteries or umbilical arteries were denuded of endothelium, perfused with control culture medium, BeWo cells or BeWo-conditioned medium (50% (v/v)) and tied off. Some vessels were frozen immediately (0 h) and others were cultured and frozen after 24 h, 48 h, 72 h or 96 h (umbilical arteries only). After cryosectioning, the number of TUNEL-positive SMC per vessel section was quantified by fluorescence microscopy (excluding cells in the two layers closest to the lumen to omit BeWo cells or residual endothelial cells from the counts). A) Spiral arteries (mean±SEM). Six sections were counted per vessel, in each of 3 (BeWo cells), or 4 (control, BeWo-conditioned medium) independent experiments. * $p<0.05$, versus control vessel at corresponding time point, Kruskal-Wallis test, Dunn's post-hoc test. B) Umbilical arteries (mean±SEM). Six sections were counted per vessel, in each of 5 independent experiments. * $p<0.05$ and ** $p<0.01$, versus control vessel at corresponding time point, Kruskal-Wallis test, Dunn's post-hoc test.

Figure 3: BeWo express Fas ligand and TRAIL

BeWo cells were cultured, fixed and stained with antibodies against Fas ligand or TRAIL. (A) FasL-positive cells and (B) TRAIL-positive cells are labelled with FITC (green); nuclei are counterstained with DAPI (blue). Pictures are representative of n=3 independent experiments.

Figure 4: BeWo release soluble Fas ligand to induce apoptosis of SMC *in vitro*

A) HASMC were incubated with unconditioned trophoblast medium (control) or BeWo-conditioned medium (CM; 50% (v/v)) for 2, 4 or 24 h. The percent of cells externalising phosphatidylserine was quantified using annexin V labelling and flow cytometry. Values were obtained by analysing 3 different SMC cultures in each of 4 separate experiments. * $p<0.05$, Kruskal-Wallis test, Dunn's post-hoc test (mean±SEM). B) HASMC were incubated for 2 h with serum-free unconditioned trophoblast medium (control) or serum-free BeWo-conditioned medium (50% (v/v); pre-treated for 1 h with either NOK-2 or an IgG isotype control (10 µg/ml). The percent of cells externalising phosphatidylserine was quantified using annexin V labelling and flow cytometry. Values were obtained by analysing 3 different SMC cultures in each of 4 separate experiments. ** $p<0.01$, repeated measures ANOVA, Bonferroni's post-hoc test (mean±SEM).

Figure 5: BeWo do not utilise TRAIL to induce SMC apoptosis *in vitro*

A) CellTracker-labelled SMC were co-cultured with BeWo cells in the presence of serum for 48h. The percent of SMC externalising phosphatidylserine was quantified using annexin V labelling and flow cytometry. Values were obtained by analysing 3 different co-cultures in each of 4 separate experiments. *** $p<0.001$, paired Student's *t* test (mean±SEM). B) CellTracker-

555 labelled SMC were co-cultured with BeWo cells in serum-free conditions in the presence or absence of TRAIL receptor blocking antibodies. After 48h, the percent of SMC externalising phosphatidylserine was quantified using annexin V labelling and flow cytometry. Values were obtained by analysing 3 different co-cultures in each of 3 separate experiments. * $p<0.05$, Kruskal-Wallis test, Dunn's post-hoc test (mean±SEM).

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Figure 6: Culture of SMC on laminin protects against Fas-induced apoptosis

HASMC were cultured on uncoated culture plates, or plates coated with collagen I, collagen IV, elastin, Matrigel, fibronectin, or laminin. Cells were grown in serum-free medium in the absence (control) or presence of a Fas-activating monoclonal antibody (1µg/ml) to induce apoptosis.
565 After 48h, the percent of SMC externalising phosphatidylserine was quantified using annexin V labelling and flow cytometry. Values were obtained by analysing 3 different co-cultures in each of 5 separate experiments. * $p<0.05$, Kruskal-Wallis test, Dunn's post-hoc test (mean±SEM).

Figure 7: BeWo cells express MMP-12

570 A) MMP-12 expression by BeWo was assessed by immunoblot analysis. Recombinant human MMP-12 was used as a positive control. An auto-radiograph representative of n=3 experiments is shown. B) BeWo cells were cultured, fixed and stained with an antibody against the catalytic domain of MMP-12 (green); nuclei were counterstained with DAPI (blue). Pictures are representative of n=3 independent experiments. C) Staining with IgG control primary antibody.

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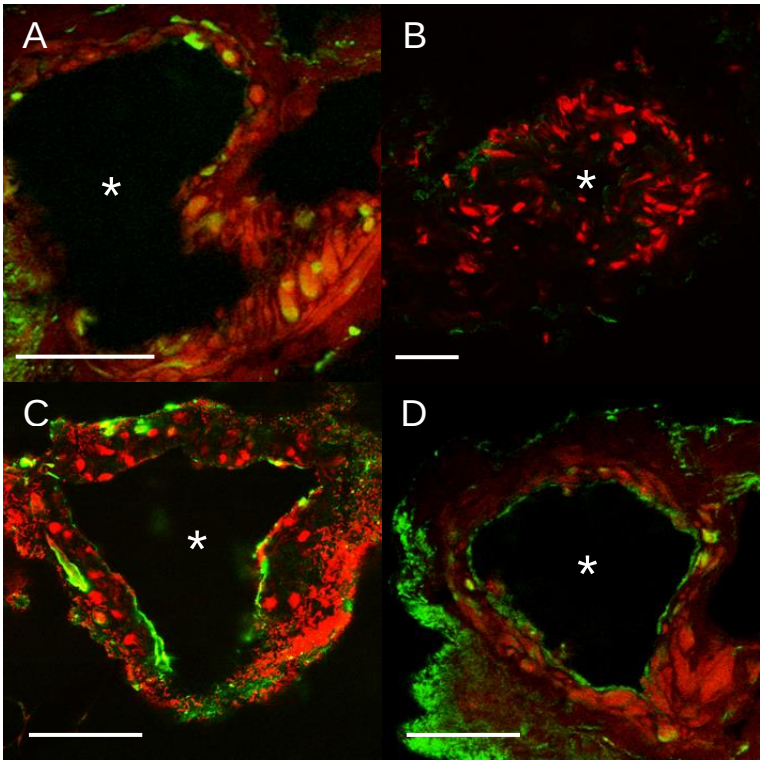
Figure 8: Trophoblast-derived factors increase MMP-12 expression in spiral artery SMC

HASMC were cultured with 1) unconditioned trophoblast medium (control), 2) BeWo-conditioned medium (50% (v/v)), 3) Fas-activating antibody or 4) etoposide (10µM) for 24 h. MMP-12 expression in these cells was assessed by immunoblot analysis. Recombinant human MMP-12 was used as a positive control. An auto-radiograph representative of n=3 experiments is shown. B, C) Spiral artery segments (minus endothelium) were perfused with B) unconditioned medium (control) or C) BeWo-conditioned medium (50% (v/v)) for 24 h. Vessels were frozen, sectioned and stained with an antibody against the catalytic domain of MMP-12 (green). Nuclei were counterstained with propidium iodide (red). * denotes the vessel lumen. Scale bar = 100µm. Pictures are representative of n≥4 independent experiments, with each experiment performed on vessels from a different biopsy.

Figure 1



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595 **Figure 2**

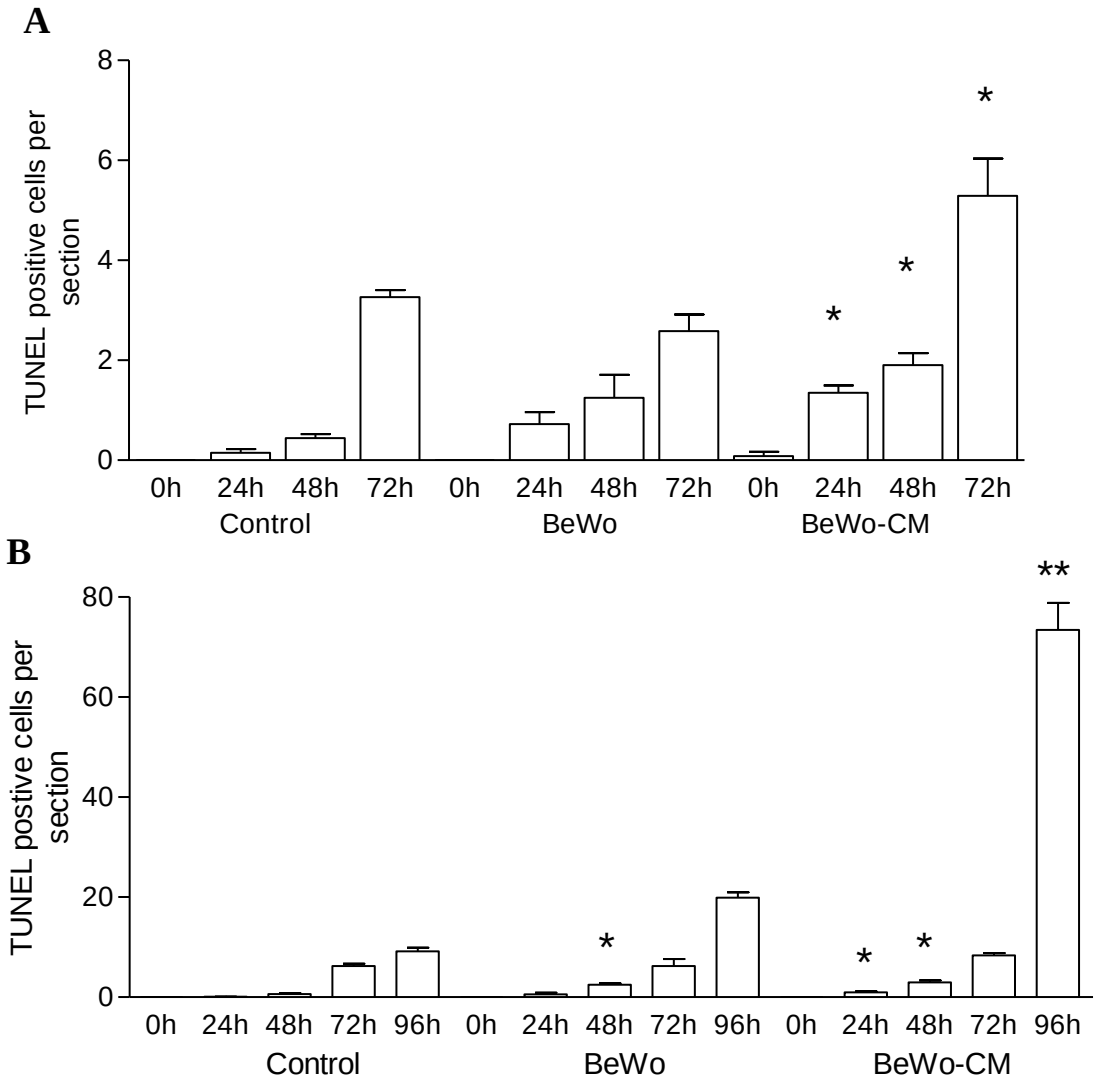

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Figure 3

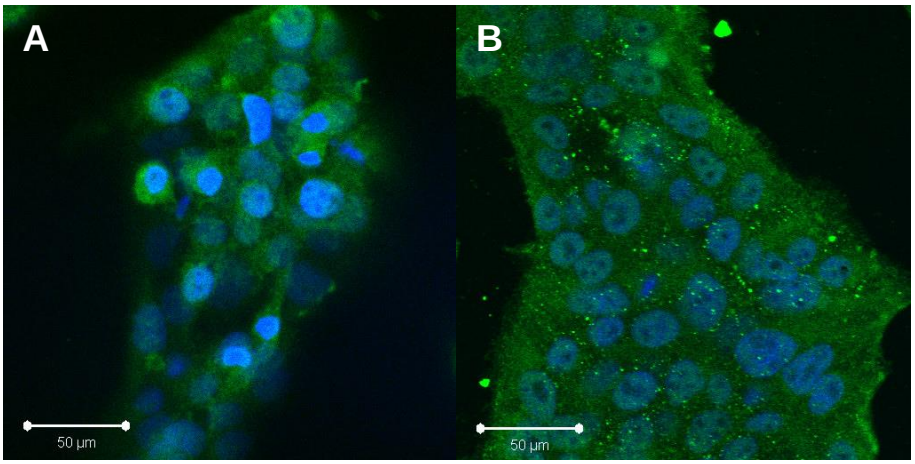

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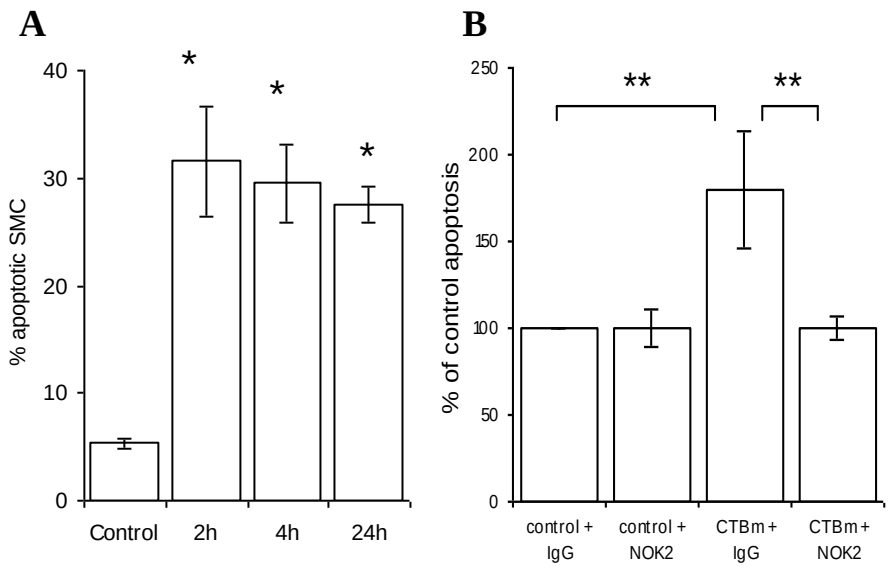
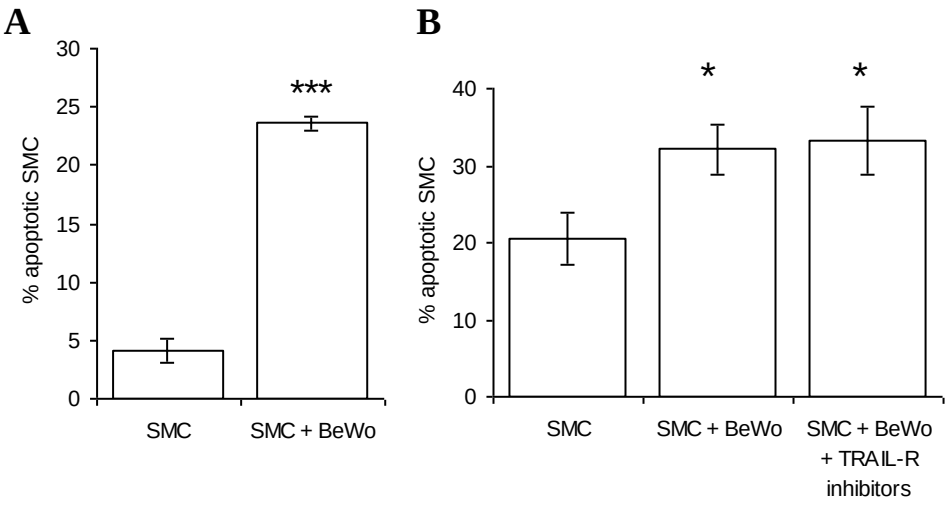
Figure 4


A
B
% apoptotic SMC
40
30
20
10
0
*
*
*
Control
2h
4h
24h
% of control apoptosis
250
200
150
100
50
0
**
**
control + IgG
control + NOK2
CTBm + IgG
CTBm + NOK2

610

Figure 5


A
% apoptotic SMC
30
25
20
15
10
5
0
***
SMC
SMC + BeWo
B
% apoptotic SMC
40
30
20
10
0
*
*
SMC
SMC + BeWo
SMC + BeWo + TRAIL-R inhibitors

615

Figure 6

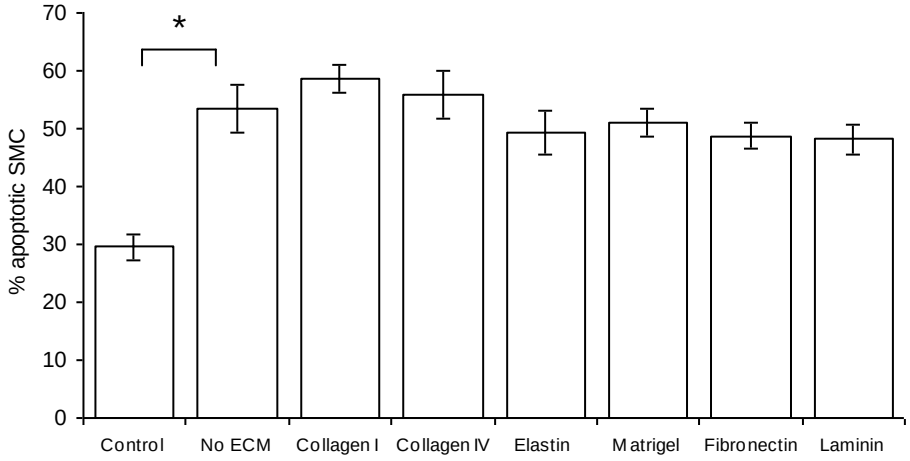

70
60
50
40
30
20
10
0
% apoptotic SMC
*
Control
No ECM
Collagen I
Collagen IV
Elastin
Matrigel
Fibronectin
Laminin

Figure 7

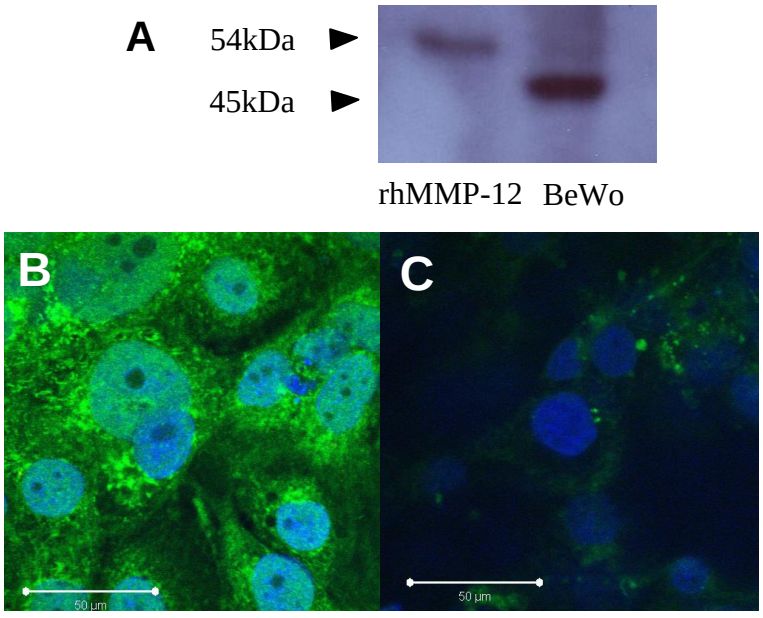


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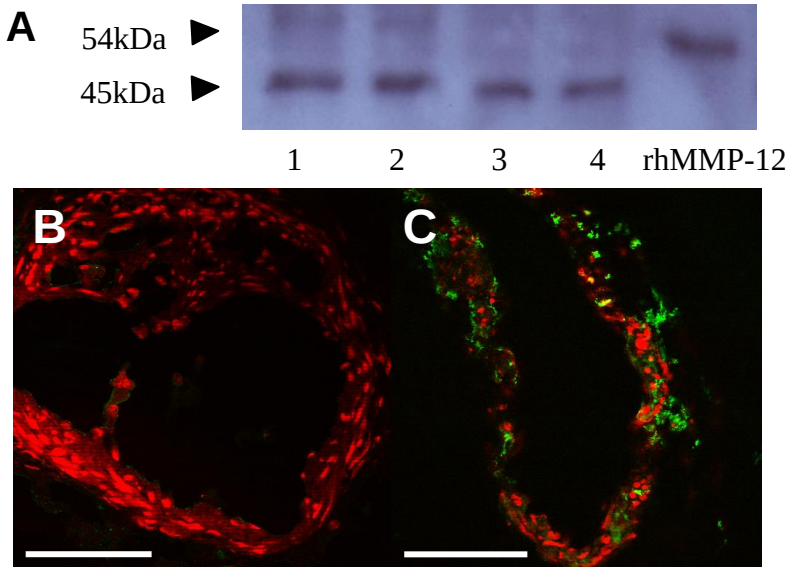
Figure 8



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